VERIFICATION OF TRANSLATION

I, OYAMA Seichiro, of 1-64-205 Higashiyama, Hirakata, Osaka, Japan, hereby declare as follows:

1. I am a translator and familiar with both the English and Japanese languages.

2. I am the translator of the document attached hereto, and certify, to the best of my knowledge and belief, that it is a true translation of Japanese Language Application No. 107512/2004.

Dated this Seventh day of December 2006.

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[TITLE OF DOCUMENT] CLAIMS

[CLAIM 1]

A breeding method of lipid producing fungi that belong to genus *Mortierella*,

said method comprising an expression suppressing step of suppressing expression of a specific gene in the lipid producing fungi.

[CLAIM 2]

A method as set forth in claim 1, wherein said expression suppressing step includes an RNAi step of suppressing expression of the specific gene by an RNAi method.

[CLAIM 3]

A method as set forth in claim 2, wherein said RNAi step includes a transformation step of introducing a recombinant expression vector into the lipid producing fungi, wherein the recombinant expression vector causes expression of double stranded RNA corresponding to all of or part of a nucleotide sequence of the specific gene.

[CLAIM 4]

A method as set forth in claim 3, wherein said RNAi step further includes an expression vector constructing step of constructing the recombinant expression vector.

[CLAIM 5]

Δ5 fatty acid desaturase, Δ6 fatty acid desaturase, Δ8 fatty acid desaturase, Δ9 fatty acid desaturase, Δ12 fatty acid desaturase, Δ15 fatty acid desaturase, Δ17 fatty acid desaturase, and ω3 fatty acid desaturase.

[CLAIM 12]

A breeding kit for carrying out the method of any one of claims 1 through 11.

[CLAIM 13]

A breeding kit as set forth in claim 12, which includes at least one of:

(a) a recombinant expression vector for causing expression of double stranded RNA corresponding to all of or part of a nucleotide sequence of the specific gene;

(b) a reagent for constructing the recombinant expression vector of (a);

(c) a reagent for introducing the recombinant expression vector of (a) into lipid producing fungi; and

(d) a reagent for culturing the lipid producing fungi and/or a transformant strain into which the recombinant expression vector of (a) have been introduced.

[CLAIM 14]

Lipid producing fungi obtained by the method or the breeding kit as defined in any one of claims 1 through 13.

[CLAIM 15]

[TITLE OF DOCUMENT] SPECIFICATION

[TITLE OF THE INVENTION] BREEDING METHOD OF LIPID PRODUCING FUNGI AND USE OF SUCH A METHOD

[TECHNICAL FIELD]

[0001]

The present invention relates to a breeding method of lipid producing fungi and use of such a method. The invention particularly relates to a method for breeding lipid producing fungi whereby gene expression is suppressed by transforming lipid producing fungi that belong to genus *Mortierella*, and use of such a method.

[PRIOR ART]

[0002]

There have been ongoing developments and actual applications of techniques for producing useful compounds through metabolism of microorganisms (broadly, fermentation techniques). In one specific example, lipid producing fungi are known that have the ability to produce a large amount of lipids through metabolism. Representative examples of such lipid producing fungi include *Mortierella alpina* and other species of genus *Mortierella*. The *Mortierella* are known to produce arachidonic acid and other polyunsaturated fatty acids (PUFA), and for this reason *Mortierella* are highly

[0005]

(1) Method employing mutation

In a population preparing step employing mutation, a population of microorganisms is prepared by introducing mutation in a variety of ways. However, the mutation occurs randomly and many different types of mutations result. As such, while it may be possible to obtain a breed (strain) with a target trait in the screening step, there is always a possibility that unexpected damage is caused on genes other than the genes associated with the target trait. For example, in the case of the lipid producing fungi, a change in the type of produced lipid may accompany reduced proliferating ability or spore forming ability, among other things. Therefore, it is not necessarily the case that a strain with good productivity is obtained in the population preparing step employing mutation.

[0006]

Further, in the method employing mutation, many different kinds of mutations randomly occur in the individuals making up the population. Thus, if a suitable screening method is not available, it takes a tremendous effort to find a mutant (strain) with a target trait because, in this case, all individuals of the population need to be screened for each different kind of mutation.

method. In these techniques, a uracil auxotrophic strain is used as a transformed host strain, and a transformed strain is screened for by using the complementary gene as a marker gene.

[0010]

As to a transformation method of (b) *M. alpina*, a technique disclosed in Non-Patent Document 4 has been known. In this technique, the spore is turned into a protoplast, and genes are introduced into the cell by an electroporation method. For the screening of transformants, hygromycin B resistance gene (hpt) from *E. coli* is used as a marker gene, and transformants that can grow in a hygromycin-containing medium are screened for.

[0011]

In modifying genetic characteristics of useful organisms to more desirable characteristics, there are cases where functions of specific genes are intentionally removed either partially or completely. While this can be achieved by the method employing mutation as described in section (1) above, the method poses some disadvantages as noted above. Therefore, there is a need for a technique of breeding mutant strains with the method employing transformation as described in section (2) above, so that functions of specific genes can be partially or completely

333-337, 1996

[Non-Patent Document 3]

Armaleo, D. et al. Curr. Genet., 17, 97-103, 1990

[Non-Patent Document 4]

Mackenzie D. A. et al. Appl. Environ. Microbiol., 66, 4655-4661, 2000

[DISCLOSURE OF THE INVENTION]

[PROBLEMS TO BE SOLVED BY THE INVENTION]

[0013]

However, there has been no report as to a method of suppressing expression of specific genes in lipid producing *Mortierella*.

[0014]

Further, while the gene expression repressing effects of the RNAi method have been well-documented in many organisms for example, whether or not the method will be effective in a specific organism cannot be known until the method is actually carried out. For example, there has been no report that suggests the effectiveness of the RNAi method in lipid producing *Mortierella*.

[0015]

Many PUFAs are essential fatty acids, and are involved in complex physiological functions in the body. Thus, the importance of PUFA as an important nutrient

[0018]

Specifically, the invention provides industrially useful method or substances as defined in (1) through (15) below.

[0019]

(1) A breeding method of lipid producing fungi that belong to genus *Mortierella*, the method including an expression suppressing step of suppressing expression of a specific gene in the lipid producing fungi.

[0020]

(2) A method as set forth in (1), wherein the expression suppressing step includes an RNAi step of suppressing expression of the specific gene by an RNAi method.

[0021]

(3) A method as set forth in (2), wherein the RNAi step includes a transformation step of introducing a recombinant expression vector into the lipid producing fungi, wherein the recombinant expression vector causes expression of double stranded RNA corresponding to all of or part of a nucleotide sequence of the specific gene.

[0022]

(4) A method as set forth in (3), wherein the RNAi step further includes an expression vector constructing

(11) A method as set forth in (9), wherein the gene that encodes the fatty acid desaturase is a gene that encodes an enzyme selected from the group consisting of: Δ5 fatty acid desaturase, Δ6 fatty acid desaturase, Δ8 fatty acid desaturase, Δ9 fatty acid desaturase, Δ12 fatty acid desaturase, Δ15 fatty acid desaturase, Δ17 fatty acid desaturase, and ω3 fatty acid desaturase.

[0030]

(12) A breeding kit for carrying out the method of any one of (1) through (11).

[0031]

(13) A breeding kit as set forth in (12), which includes at least one of:

(a) a recombinant expression vector for causing expression of double stranded RNA corresponding to all of or part of a nucleotide sequence of the specific gene;

(b) a reagent for constructing the recombinant expression vector of (a);

(c) a reagent for introducing the recombinant expression vector of (a) into lipid producing fungi; and

(d) a reagent for culturing the lipid producing fungi and/or a transformant strain into which the recombinant expression vector of (a) have been introduced.

[0032]

the lipids it produces.

[0036]

The *Mortierella* are well known lipid producing fungi, and include highly reliable species such as *M. alpina.* Thus, with the present invention, a strain with improved lipid productivity, modified lipid composition, and/or improved productivity of specific lipids can be produced both easily and efficiently.

[BEST MODE FOR CARRYING OUT THE INVENTION]

[0037]

The following will describe one embodiment of the present invention. It should be noted however that the invention is not limited in any way by the following description.

[0038]

The present invention provides a method including an expression suppressing step of suppressing expression of specific genes, particularly genes involved in lipid metabolism, whereby lipid producing fungi with desirable characteristics (traits) are bred. The invention also provides use of such a breeding method.

[0039]

The following will describe *Mortierella*, a breeding method, and use of the method in this order, according to

industries producing PUFA.

[0042]

The source of *M. alpina* or other *Mortierella* are not particularly limited. For example, the fungi can be obtained from various microorganism depositary institutions such as the Institute for Fermentation or ATCC (American Type Culture Collection). In the case of strains for which patent applications have been filed, the organisms can be obtained from the International Patent Organism Depositary in the National Institute of Advanced Industrial Science and Technology. Alternatively, an unknown strain of *Mortierella* may be obtained from natural environment by a known screening method.

[0043]

[2] Breeding method of lipid producing fungi according to the present invention

A breeding method according to the present invention is not particularly limited as long as it includes the expression suppressing step. As such, the method is not just limited to particular steps, materials, or conditions. As used herein, the "expression suppressing step" is not particularly limited as long as expression of specific genes in the lipid producing fungi is suppressed. As such, the step can be carried out by conventional

[0046]

[2-1] RNAi step

The RNAi step according to the present invention is not particularly limited as long as expression of specific genes in lipid producing fungi is suppressed by an RNAi method. As such, the step is not just limited to particular methods, conditions, or materials.

[0047]

As used herein, the "RNAi method" refers to a method in which gene expression is suppressed by a phenomenon known as RNAi. The "RNAi" refers to the situation where the presence of double stranded RNA ("dsRNA") in the cell promotes degradation of mRNA that hybridizes with the dsRNA, and thereby suppresses expression of genes corresponding to the mRNA.

[0048]

Therefore, the RNAi step can be regarded as a step of introducing dsRNA complementary to the nucleotide sequence that codes for the entire part or some part of a gene whose expression is to be suppressed. The nucleotide sequence or length of introduced RNA is not particularly limited as long as it can suppress expression of a target gene. For this purpose, techniques of conventional RNAi methods can be suitably used.

vector is constructed in *Mortierella* such that the recombinant expression vector, with a suitable sequence of DNA ligated under the control of a promoter, brings about expression of double stranded RNA that causes RNAi. In other words, in the expression vector constructing step, a recombinant expression vector is constructed in such a manner that a gene that encodes the double stranded RNA is expressed under the control of a promoter.

[0051]

The type of gene whose expression should be suppressed is not particularly limited and is suitably selected from genes of *Mortierella* depending on its purpose. As to specifics of the gene, detailed description will be given in conjunction with the transformation step in section [2-1-2] below.

[0052]

The method of constructing a recombinant expression vector for RNAi is not particularly limited, and conventional methods can be used therefor. For example, gene cassettes ligated via a linker sequence or other sequences are transcribed *in vivo* in such a manner that both the sense strand and antisense strand of a gene whose expression should be suppressed are transcribed,

[0055]

Further, the length of the nucleotide sequence of the double stranded RNA is not particularly limited as long as it can efficiently cause RNAi. Further, in the case of double stranded RNA corresponding to only some part of the gene, a portion of the gene is suitably selected depending on purpose.

[0056]

The promoter is not particularly limited either as long as it can effectively express the gene that encodes the double stranded RNA in *Mortierella* (lipid producing fungi). As such, conventional promoters can be suitably used. A non-limiting example of such conventional promoters is hisH4.1 promoter.

[0057]

Various types of conventional vectors can be used to provide the recombinant expression vector. Some of the examples include a plasmid, phage, and cosmid, which are suitably selected according to the type of host cell (type of *Mortierella*) or the employed method of introduction. Specific examples include pBR322, pBR325, pUC-type, pBluescript-type, and pBI-type vectors.

[0058]

The recombinant expression vector may include DNA

above (medium without specific nutrients) can be easily screened for transformed strains that have incorporated the recombinant expression vector. Specific examples of drug-resistant genes include genes resistant to ampicillin, hygromycin, bleomycin, kanamycin, gentamicin, or chloramphenicol. In this way, transformed strains that have incorporated the recombinant expression vector can be selected by simply screening an antibiotic-containing medium for the strains that grow in the medium.

[0061]

The enhancer is not particularly limited as long as it is a DNA nucleotide sequence that promotes transcription of an adjacent gene on a single DNA strand, i.e., in a cis position. For example, a repeating sequence of 72 base long near the replication origin of monkey-derived simian virus 40 (SV 40) can be used as an enhancer. Other conventional enhancers can be used as well. With the use of an enhancer, the transcription activity can be improved even when the promoter region alone is insufficient to obtain a sufficient level of desired gene expression. As described above, various types of DNA segments can be included in the recombinant expression vector depending on intended use of the vector or the type of vector-introduced cell.

particularly limited, and can be selected from commercially available products.

[0064]

The proliferation method (producing method) of the recombinant expression vector is not particularly limited, and conventional methods can be used therefor. Generally, *Escherichia coli* are used as a host to proliferate the vector. Here, *Escherichia coli* may be suitably selected according to the type of vector used.

[0065]

[2-1-2] Transformation step

The transformation step according to the present invention is not particularly limited as long as the recombinant expression vector for expressing double stranded RNA corresponding to the entire part or some part of the nucleotide sequence of a specific gene is introduced into the lipid producing fungi. As such, the procedure, condition, or material used in the step is not particularly limited. That is, in the transformation step, the recombinant expression vector constructed in the expression vector constructing step is introduced into the lipid producing fungi (transformation).

[0066]

By carrying out the transformation step, a large

Δ8 fatty acid desaturase gene, Δ9 fatty acid desaturase gene (GB accession No. AJ278339, AF085500, Y18554, Y18553, AB015612, AB015611), Δ12 fatty acid desaturase gene (GB accession No. AF417244, AF110509, AB020033, Eur. J. Biochem. 261, 812-820 (1999)), Δ15 fatty acid desaturase gene, Δ17 fatty acid desaturase gene, and ω3 fatty acid desaturase gene. By suppressing expression of genes involved in lipid metabolism, the yield of desired lipids can be increased or decreased. Further, the type or composition of produced lipids can be modified.

[0068]

In the Examples below, MAELO gene and Δ12 fatty acid desaturase gene are used as examples of genes involved in lipid metabolism.

[0069]

The MAELO gene is known to exhibit a weak activity for generating dihomo-γ-linolenic acid (DGLA, 20:3n-6) via γ-linolenic acid (GLA, 18:0n-6) (Japanese PCT Laid-Open Publication No. 2002-523098). However, it is believed that a gene that plays a principal role in this reaction is the GLELO gene, which has been isolated. As such, other functions of the MAELO gene are suspected. According to an annotation of the GenBank, a role of the MAELO gene in the catalysis of the chain-elongating reaction of

RNAi method as in the Examples below, a strain (mutant strain) can be obtained in which a proportion of ω9 fatty acids, such as mead acid, is increased.

[0072]

The present invention is applicable even when a gene whose expression is to be suppressed is not specified. For example, when only a nucleotide sequence of EST with unknown functions is known, an expression vector that encodes double stranded RNA corresponding to the EST is constructed. By obtaining transformant strains with the expression vector and analyzing functions of the transformed strains, functions of the gene with the EST can be analyzed. In this manner, with a breeding method of the present invention, unknown functions of a gene can be predicted.

[0073]

A transformation method (gene introducing method) used in the transformation step is not particularly limited, and conventional methods such as an electroporation method or particle delivery method can be suitably used. When using an electroporation method in *Mortierella*, it is preferable that the fungi be used in the form of protoplasts. A non-limiting example of the particle delivery method is a particle gun method. In the Examples

invention is to provide a method for breeding a novel strain of desired characteristics (traits) by suppressing expression of a predetermined gene in *Mortierella* (lipid producing fungi). Accordingly, the present invention is not just limited to the individual procedures of transformation, culturing, and selection as specifically described above. Instead, the invention also includes a breeding method employing other procedures.

[0077]

[3] Use of the present invention

[3-1] Lipid producing fungi (novel breed)

As described above, a breeding method of lipid producing fungi according to the present invention is adapted to suppress expression of a specific gene and thereby provide a breed with improved characteristics (traits). Thus, with the method, a novel breed (novel strain) can be efficiently and effectively produced by using lipid producing fungi of genus *Mortierella* as an original breed. The *Mortierella* are well known lipid producing fungi, and include highly reliable species such as *M. alpina*. Thus, for example, a strain with improved lipid productivity can be produced both easily and efficiently.

[0078]

Further, with a breeding method according to the

useful, and a transformant strain obtained by a breeding method of the present invention also belongs to the present invention. Use of the present invention also includes, for example, a breeding kit for implementing the invention, as described below.

[0081]

[3-2] Breeding kit

A breeding kit according to the present invention is not particularly limited as long as it is for implementing the breeding method described in section [2] above. As such, specific structures, materials, devices, and the like included in the breeding kit are not particularly limited. Specifically, a breeding kit according to the present invention is adapted to carry out the respective steps of the breeding method.

[0082]

For example, in order to carry out the transformation step, the breeding kit may include (a) a recombinant expression vector for expressing double stranded RNA corresponding to the entire part or some part of the nucleotide sequence of a specific gene. Further, in order to carry out the expression vector constructing step, the breeding method may include (b) reagents for constructing the recombinant expression vector (a). Further, in order to

breeding method according to the present invention can be carried out both easily and reliably. Note that, since the breeding kit is for carrying out the breeding method of the present invention, a novel breed of lipid producing fungi obtained with the use of the breeding kit is also included in the present invention.

[0085]

[3-3] Producing method of lipid, and lipids obtained by the producing method

In a producing method of lipids according to the present invention, PUFA-containing lipids are produced from the lipid producing fungi described in section [3-1] above. For example, PUFA-containing lipids can be conveniently produced by culturing the lipid producing fungi.

[0086]

In the following, lipids produced by *Mortierella* are described.

[0087]

The PUFA-containing lipids are one of many useful products produced by *Mortierella*. A wild-type strain with good PUFA productivity is known to produce a large amount of ω6 PUFA that contains mostly arachidonic acid, and very long-chain saturated fatty acids (VLSA) are also

containing the same number of carbon atoms. For example, by comparing fatty acids containing 18 carbon atoms, oleic acid and linoleic acid, which are monounsaturated fatty acid and diunsaturated fatty acid, respectively, have melting points of 13.4°C and -5.2°C, which are significantly lower than the melting point 69.6°C of stearic acid, an example of saturated fatty acids. As to VLSA, these fatty acids have considerably high melting points, since the melting point increases with an increasing chain length. Docosanoic acid (behenic acid), tetracosanoic acid (lignoceric acid), hexacosanoic acid (cerotic acid) have melting points 79.9°C, 84.2°C, and 87.7°C, respectively. The melting point of lipids such as triglycerides is strongly influenced by the melting point of the constituting fatty acids, and therefore the melting point of oil or fat containing VLSA is generally high. For this reason, an oil or fat is often a solid at ordinary temperature, or may deposit and turn turbid or solidify when stored at a low temperature. For the separation and removal of a solid fat to obtain a liquid oil, procedures employing a wintering method or emulsion separation method are commonly used (Oils and Fats Handbook, SAIWAI SHOBO (1988) P. 261). An oil or fat produced by *Mortierella* contains several % VLSA, through the

desaturase activity leads to accumulation of dihomo-γ-linolenic acid.

[0093]

In this manner, while different PUFAs can be produced by mutational modification, a mutant strain cannot be obtained without damaging genes of the PUFA-producing wild-type strain other than the genes associated with the target traits.

[0094]

These problems can be solved by a lipid producing method according to the present invention. That is, the lipid producing method according to the present invention can reduce the amount of VLSA-containing lipids, which easily solidify to form fats. Therefore, with the method, liquid oil suitable for food can be produced without time-consuming and costly procedures such as the wintering method or emulsion separation method.

[0095]

Further, because the lipid producing method does not favor the alternative VLSA biosynthesis pathway, a target PUFA can be produced with improved productivity. Further, a PUFA-producing strain can be obtained without damaging genes other than the gene associated with desired traits.

containing ω9 PUFA in a proportion of not less than 8% with respect to total fatty acids of the lipids.

- Lipids produced by the lipid producing method, containing mead acid in a proportion of not less than 1.3% with respect to total fatty acids of the lipids.

- Lipids produced by the lipid producing method, containing arachidonic acid in a proportion of not less than 10% with respect to total fatty acids of the lipids, and very long-chain saturated fatty acids in a proportion of not more than 0.1% with respect to total fatty acids of the lipids.

[0100]

While the invention is susceptible to various modifications and alternative forms, a specific embodiment thereof will be described below in more detail by way of Examples. It should be understood, however, that it is not intended to limit the invention to the particular forms disclosed, but on the contrary, the invention is to cover all modifications, equivalents, and alternatives falling within the scope of the invention as defined in the appended claims.

[Examples]

[0101]

(Example A) Functional analysis of MAELO gene

cerevisiae INVSc1 strain, and strains that grew on a uracil-deficient plate (2% glucose, 0.17% Yeast Nitrogen Base w/o Amino Acids and Ammonium Sulfate (Difco), 0.5% ammonium sulfate, histidine (20 mg/l), leucine (60 mg/l), tryptophan (40 mg/l), 2% Bacto agar) were selected as transformed strains. These strains were then inoculated once with a platinum loop on a medium containing 2% raffinose, 0.17% Yeast Nitrogen Base w/o Amino Acids and Ammonium Sulfate (Difco), 0.5% ammonium sulfate, 1% Tergitol Type NP-40, histidine (20 mg/l), leucine (60 mg/l), tryptophan (40 mg/l), and 0.05% stearic acid (18:0), and the medium was cultured for 6 hours by shaking. Then, 2% (w/v) galactose was added to the medium so as to induce expression of MAELO gene ligated downstream of the GAL1 promoter in the plasmid pY2MEL, and the medium was cultured at 28°C for 42 hours by shaking. The fungi were collected by centrifugation, and freeze-dried. After inducing the fatty acid residue in the fungi to methyl ester by a hydrochloric acid/methanol method, the sample was extracted with hexane. The resulting fatty acid methyl ester after the removal of hexane was analyzed by gas chromatography. Table 1 below represents the yields of very long-chain saturated fatty acids in each broth.

constructed. Then, by using plasmid pD4 (D. A. Mackenzie et al. Appl. Environ. Microbiol., 66, 4655-4664, 2000) as a template, PCR was carried out with LA Taq (TaKaRa). As the primers, primer HisProFX and primer TrpCRX were used. The reaction was carried out in 30 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes. The amplified DNA fragments were digested with restriction enzyme EcoRI, and the resulting DNA fragments were inserted at the EcoRI site of the plasmid pBluescriptIISK+(BamHI-), so as to construct plasmid pBlueHpt.

[0108]

HisProFX: 5'-tacgaattcaagcgaaagagagattatgaa-3' (SEQ ID NO: 1)

TrpCRX: 5'-gaagaattccctctaaacaagtgtacctgt-3' (SEQ ID NO: 2)

M. alpina was cultured at 28°C for 5 days in GY broth (2% glucose, 1% yeast extract, pH = 6.0). From the resulting fungi, genomic DNA was prepared according to the procedure of E. Sakuradani et al. Eur J. Biochem., 260, 208-216, 1999.

[0109]

By using the genomic DNA of *M. alpina* as a template, PCR was carried out with LA Taq (TaKaRa). As the primers,

fragment of about 0.7 kb was inserted in the NcoI-BlnI site of the plasmid pBlueMEi3, so as to construct plasmid pBlueMEi5.

[0111]

MAELORNAi1: 5'-ttggatccatggccgccgcaatcttggaca-3' (SEQ ID NO: 5)

MAERORNAi5: 5'-tgatctcctaggtggaacactgatagccac-3' (SEQ ID NO: 6)

A fragment of about 3.3 kb obtained by digesting the plasmid pBlueMEi5 with the restriction enzyme EcoRI was inserted in the EcoRI site of plasmid pDura5, so as to construct plasmid pDura5MEi51.

[0112]

By using the genomic DNA of *M. alpina* as a template, PCR was carried out with LA Taq (TaKaRa). As the primers, primer MAELORNAi1-1 and primer MAELORNAi2 were used. The reaction was carried out in 30 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. The amplified DNA fragment of about 0.7 kb was then TA cloned into the pT7Blue T-Vector (TaKaRa). After confirming the nucleotide sequence, the fragment was digested with restriction enzymes NcoI and BamHI. The resulting DNA fragment of about 0.7 kb was then inserted in the NcoI-BamHI site of the plasmid pBlueHpt, so as to

stranded RNA corresponding to about 700 bp of the MAELO gene. In the case of plasmid pDura5Mei41, double stranded RNA corresponding to about 500 bp of the MAELO gene can be expressed in excess.

[0115]

Using the plasmid pDura5MEi51 or pDura5Mei41, transformation was carried out by a particle delivery method. As the host, *M. alpina* Δura-1 strain (Δura5) was used. For the selection of transformed strains, SC agar medium was used (5.0 g of Yeast Nitrogen Base w/o Amino Acids and Ammonium Sulfate (Difco), 1.7 g of $(NH_4)_2SO_4$, 20 g of glucose, 20 mg of adenine, 30 mg of tyrosine, 1.0 g of methionine, 2.0 mg of arginine, 2.0 mg of histidine, 4.0 mg of lysine, 4.0 mg of tryptophan, 5.0 mg of threonine, 6.0 mg of isoleucine, 6.0 mg of leucine, 6.0 mg of phenylalanine, and agar (20 g/l)).

[0116]

Several ten transformed strains obtained for each plasmid were inoculated on GY medium. For each plasmid used, two of the transformed strains which stably held the marker ura5 gene were selected. Specifically, transformed strains #1 and #2 were used for cultures transformed by pDura5Mei51, and transformed strains #3 and #4 were used for cultures transformed by pDura5Mei41. The

NO: 8)

Each transformed strain was then inoculated in a test tube containing 10 ml of GY broth. The sample was cultured at 28°C for 12 days by shaking, and the fungi were collected by filtration.

[0118]

From a removed portion of fungi, total RNA was extracted using the RNeasy plant mini kit (QIAGEN). Then, a reverse transcription reaction was carried for 1 μg of total RNA, using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen), so as to synthesize cDNA. For the reaction, a random hexamer was used as a primer. By using 1 μg of cDNA as a template, PCR was carried out with primer MAELO-1, primer MAELO-2, and ExTaq (TaKaRa). The reaction was carried out in 20 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute.

[0119]

Primer: MAELO-1: 5'-agtccatcgactccttcgtcttcca-3' (SEQ ID NO: 9)

Primer: MAELO-2: 5'-cggtgtcagccaactcccagtactt-3' (SEQ ID NO: 10)

The PCR product was electrophorased, and fluorescence intensities were compared between the bands

[0121]

As can be seen from Table 2, the proportion of very long-chain saturated fatty acids dropped significantly in the transformed strains. Specifically, synthesis of very long-chain saturated fatty acids was completely suppressed in the transformed strains #1 and #2, whereas their synthesis was partially suppressed in the transformed strains #3 and #4.

[0122]

(Example C) Breeding of Δ12 fatty acid desaturase gene expression suppressing strain

For the excess expression of double stranded RNA corresponding to a portion of the Δ12 fatty acid desaturase gene, a vector was constructed according to the following procedure.

[0123]

By using plasmid pMOD10 (Eur. J. Biochem. 261, 812-820 (1999)) as a template, PCR was carried out with primer Δ12-1, primer Δ12-2, and LA Taq (TaKaRa), so as to amplify a DNA fragment of about 670 bp. The reaction was carried out in 30 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute.

[0124]

Primer Δ12-1: 5'-gcggatccatggcacctcccaacacta-3'

the *M. alpina* Δura-1 strain, using a particle delivery method. For the selection of transformed strains, strains that can grow in SC agar medium were selected. Several ten transformed strains were inoculated on GY medium, and strains that stably held the marker ura5 gene were selected. As in Example B, it was confirmed that three of the selected strains (transformed strains #5 through #7) had incorporated the plasmid pDura5Δ12RNAi, through recombination, in the 18SrDNA region of the chromosome. Each transformed strain was cultured at 28°C for 5 days in a flask containing 500 ml of SC broth. For the breeding of the Δura-1 strain (control), uracil was added (50 mg/l) to the SC broth.

[0126]

From a removed portion of the fungi, cDNA was synthesized according to the procedure of Example B, and PCR was carried out using primer Δ12-3, primer Δ12-4, and Ex Taq (TaKaRa). The reaction was carried out in 20 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute.

[0127]

Primer Δ12-3: 5'-ttgctattgatctgacctgggcctc-3' (SEQ ID NO: 13)

Primer Δ12-4: 5'-tgggaacaaagacctggtccttgg-3' (SEQ ID

	Control	Transformed strains		
	Δura-1	#5	#6	#7
Proportions in total fatty acids (%)				
ω6 PUFA				
linoleic acid	14.8	0.1	0.0	0.1
γ-linolenic acid	3.4	0.2	0.0	0.5
Dihomo-γ-linolenic acid	2.4	0.2	0.2	0.1
Arachidonic acid	11.9	1.5	1.1	2.3
Total of ω6 fatty acids	32.5	2.0	1.3	3.0
ω9 PUFA				
18:2 ω9	0.2	6.3	6.9	5.9
20.2 ω9	0.0	0.9	0.9	0.8
Mead acid	0.0	1.6	1.6	1.3
Total of ω9 fatty acids	0.2	8.8	9.4	8.0

[0130]

As can be seen from Table 3, the proportion of ω6 PUFA dropped in the transformed strains, but there was a significant increase in the proportion of ω9 PUFA. Specifically, while a considerable amount of mead acid was present in the transformed strains, the Δura-1 strain did not contain any mead acid. That is, it was found that the ability to produce mead acid and other ω9 PUFAs can be obtained by suppressing expression of the Δ12 fatty acid desaturase gene.

[INDUSTRIAL APPLICABILITY]

[0131]

[TITLE OF THE DOCUMENT] ABSTRACT

[ABSTRACT]

[OBJECT] To provide a breeding method for effectively and efficiently breeding lipid producing *Mortierella* by suppressing expression of specific genes. The invention also provides use of such a method.

[MEANS TO ACHIEVE THE OBJECT] A method of breeding a lipid-producing strain belonging to the genus *Mortierella*. According to the method of breeding a lipid-producing strain which involves an expression-inhibiting step of inhibiting the expression of a specific gene in the lipid-producing strain as described above, a lipid-producing strain belonging to the genus *Mortierella* can be efficiently and effectively bred.

[SELECTED DRAWINGS] None